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Chapter Four

FUNCTIONAL ANALYSIS OF THE SECRETORY PRECURSOR PROCESSING MACHINERY OF *BACILLUS SUBTILUS*:

IDENTIFICATION OF A EUBACTERIAL HOMOLOG OF ARCHEAL AND EUKARYOTIC SIGNAL PEPTIDASES

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FUNCTIONAL ANALYSIS OF THE SECRETORY PRECURSOR PROCESSING MACHINERY OF *BACILLUS SUBTILIS*:

IDENTIFICATION OF A EUBACTERIAL HOMOLOGUE OF ARCHAEAL AND EUKARYOTIC SIGNAL PEPTIDASES

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Summary

About 47% of the genes of the Gram-positive eubacterium *Bacillus subtilis* belong to paralogous gene families. The present studies were aimed at the functional analysis of the *sip* gene family of *B. subtilis,* consisting of five chromosomal genes, denoted *sipS, sipT, sipU, sipV,* and *sipW.* All five *sip* genes specify type I signal peptidases (SPases), which are actively involved in the processing of secretory pre-proteins. Interestingly, strains lacking as many as four of these SPases could be obtained. As shown with a temperature-sensitive SipS variant, only cells lacking both SipS and SipT were not viable, which may be due to jamming of the secretion machinery with secretory pre-proteins. Thus, SipS and SipT are of major importance for protein secretion. This conclusion is underscored by the observation that only the transcription of the *sipS* and *sipT* genes is temporally controlled *via* the DegS-DegU regulatory system, in concert with the transcription of most genes for secretory pre-proteins. Notably, the newly identified SPase SipW is highly similar to SPases from archaea and the ER membrane of eukaryotes, suggesting that these enzymes form a sub-family of the type I SPases, which is conserved in the three domains of life.

Introduction

Since 1995, at least twelve microbial genomes have been completely sequenced and annotated (for a recent update, see the microbial database at: http://www.tigr.org/tdb/mdb/mdb.html). A remarkable common property of these genomes is that many genes are present in multiple (paralogous) copies. A good example is the genome of *Bacillus subtilis*, which contains about 4,200 genes, 47% of which belong to paralogous gene families (174). Close examination of the corresponding proteins of *B. subtilis* has indicated that many of these are involved in transcription regulation, and the transport of compounds into and out of the cell. Interestingly, most genes for components of the protein secretion machinery are present in only one copy, despite the fact that *B. subtilis* has a large capacity for protein secretion. The only known exception concerns the genes for type I signal peptidases (SPases) (105). These SPases remove amino-terminal signal peptides from secretory pre-proteins during, or shortly after their translocation across the cytoplasmic membrane, in order to release these proteins from the *trans* side of this membrane (for review, see (89)).

Homologous type I SPases have been identified in archaea, Gram-positive and Gram-negative eubacteria, the inner membrane of yeast mitochondria, the thylakoid membrane of chloroplasts, and the ***e***ndoplasmic ***r***eticular (ER) membranes of yeast and higher eukaryotes. Despite the fact that considerable similarities exist between the known type I SPases when amino acid sequences are compared pairwise, only few residues are strictly conserved in all known enzymes of this family (89). In particular, the type I SPases of eubacteria, mitochondria and chloroplasts differ considerably from their homologues in archaea and the ER membrane, indicating that these enzymes belong to distinct sub-families of SPases. In what follows, we will

refer to these sub-families as the P (***p***rokaryotic)- and ER-type SPases, respectively. Most P-type SPases contain conserved serine and lysine residues which are essential for enzymatic activity, possibly by forming a catalytic dyad (99;111-113). The putative active site serine residue of the P-type SPases is also conserved in the ER-type SPases. In contrast, the putative active site lysine residue of the P-type SPases is replaced with a histidine residue in the ER-type SPases (103;114).

In many eubacteria, such as *Escherichia coli* (185), *Haemophilus influenzae* (186), *Helicobacter pylori* (187), and *Mycobacterium tuberculosis* (188), one P-type SPase seems to be sufficient for the processing of secretory pre-proteins. Similarly, the archaea *Methanococcus jannaschii* (189) and *Methanobacterium thermoauthotrophicum* (190), and the yeast *Saccharomyces cerevisiae* (191) contain only one ER-type SPase. As shown for *E. coli* (86;192) and yeast (193), these SPases are likely to be essential for cell viability. In contrast, two paralogous P-type SPases are found in *Synechocystis* PCC 6803 (194) and the inner membrane of yeast mitochondria (195;196), while the presence of two paralogous ER-type SPases appears to be characteristic for most eukaryotic species (89). Finally, the largest numbers of paralogous SPases appear to be present in the archaeon *Archaeoglobus fulgidus*, which contains three genes for ER-type SPases (197), and the Gram-positive eubacterium *B. subtilis* in which seven *sip* genes for type I SPases have been identified. Five of the *sip* genes of *B. subtilis* (denoted *sipS*, *sipT*, *sipU*, *sipV* and *sipW*) are located on the chromosome (103;105); two additional *sip* genes (denoted *sipP*) are located on plasmids which were, thus far, only found in natto-producing strains of *B. subtilis* (107).

Thus far, the presence of both P- and ER-type SPases in one organism was only evident for eukaryotes, which contain P-type SPases in their organelles and ER-type SPases in the ER (89). In this paper, we document our surprising observation that one of the seven type I SPases of *B. subtilis* (*ie.* SipW) belongs to the ER-type sub-family of SPases. Thus, *B. subtilis* is the first organism known to contain SPases of the P- and the ER-type in one membrane. Furthermore, our studies, aimed at the functional analysis of the type I SPases of *B. subtilis*, show that SipS and SipT are the most important SPases of *B. subtilis*, whereas SipU, SipV, and SipW appear to have a minor role in precursor processing.

Materials and Methods

Plasmids, bacterial strains and media

Table 4.I lists the plasmids and bacterial strains used. TY medium (tryptone/yeast extract) contained Bacto tryptone (1%), Bacto yeast extract (0.5%) and NaCl (1%). Minimal medium for *B. subtilis* contained: 100 mM potassium phosphate buffer pH 7.0, glucose (1%), trisodium-citrate (3 mM), $MgSO_4$ (3 mM), casamino acids (0.1%), potassium-glutamate (0.2%), tryptophan (20 µg/ml), histidine (20 µg/ml), methionine (20 µg/ml), tyrosine (20 µg/ml), adenine (20 µg/ml), uracil (20 µg/ml), nicotinic acid (0.4 µg/ml), riboflavin (0.4 µg/ml), and Fe-ammoniumcitrate (1.1 µg/ml). S7 media 1 and 3, used for labeling of *B. subtilis* proteins with [^{35}S]-methionine (Amersham), were prepared as described by van Dijl *et al*. (164;165). If required, media for *E. coli* were supplemented with ampicillin (Ap; 50 µg/ml), erythromycin (Em; 100 µg/ml), or kanamycin (Km; 40 µg/ml); media for *B. subtilis* were supplemented with chloramphenicol (Cm; 5 µg/ml), Em (2 µg/ml), Km (10 µg/ml) or spectinomycin (Sp; 100 µg/ml).

DNA and RNA techniques

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described by Sambrook *et al.* (161). Enzymes were from Boehringer Mannheim. *B. subtilis* was transformed by growth in minimal medium until an optical density at 600 nm (OD600) of ± 1, the subsequent addition of plasmid or chromosomal DNA to the culture, and continued incubation for at least four hours. Multiple *sip*-mutants were constructed by transforming single mutants with chromosomal, or linearized plasmid DNA containing a disrupted *sip*-gene. To minimize the number of antibiotic resistance markers in the cells, triple *sip*-mutants were prepared from double *sip*-mutants containing no, or only one antibiotic resistance marker. Correct integration of linearized DNA fragments, or plasmids in the chromosome of *B. subtilis* was verified by Southern hybridization. PCR was carried out with Vent DNA polymerase (New England Biolabs) as described by van Dijl *et al.* (113). DNA and protein sequences were analyzed using version 6.7 of the PCGene Analysis Program (Intelligenetics Inc.). The BLAST algorithm (198) was used for protein comparisons in GenBank. RNA was isolated with the RNeasy total RNA kit from Qiagen. RNA reference markers were from Gibco BRL Life Technologies. Northern hybridizations were performed as described by Bolhuis *et al.* (104).

Table 4.I. Plasmids and Bacterial Strains

Plasmids	Relevant properties	Reference
pGDL41	Encodes pre(A13i)-β-lactamase and SipS of *B. subtilis*; replicates in *E. coli* and *B. subtilis*. 8.1 kb; Ap^r; Km^r	(103)
pGDL48	Lacks the *sipS* gene and contains a multiple cloning site; otherwise identical to pGDL41; 7.5 kb; Ap^r; Km^r	(107)
pMO	Encodes pre(A13i)-β-lactamase, used for the site-directed mutagenesis of *sipS*	(113)
pS-x	pMO derivatives carrying mutant *sipS* genes (x indicates the position and type of amino acid substitution in the corresponding mutant proteins); otherwise identical to pGDL41; 8.1 kb; Ap^r; Km^r	(113)
pKTH10	Encodes the α-amylase (AmyQ) of *B. amyloliquefaciens*, 6.8 kb; Km^r	(201)
pCY66	Encodes the PSBT domain of the *P. shermanii* transcarboxylase 1.3 S subunit	(199)
pBR322*amyQ*	Encodes the α-amylase (AmyQ) of *B. amyloliquefaciens*	This study
pBR322*amyQ*-BT	Encodes the AmyQ-PSBT fusion protein	This study
pKTH10-BT	As pKTH10, encodes the AmyQ-PSBT fusion protein, 7.0 kb	This study
pX	Vector for the integration of genes in the *amyE* locus of *B. subtilis;* integrated genes will be transcribed from the *xylA* promoter; carries the *xylR* gene; 7.5 kb; Ap^r; Cm^r	(202)
pXS	pX derivative; carries *sipS* downstream of the *xylA* promoter; 8.2 kb; Cm^r	This study
pXS-D146A	pX derivative; carries *sipS-D146A* downstream of the *xylA* promoter; 8.2 kb; Cm^r	This study
pLGW200	Integration vector for *B. subtilis* with a promoterless *lacZ* gene fused to the ribosome-binding site of the *spoVG* gene; 6.8 kb; Cm^r	(203)
pLGW201	pLGW200 derivative with a transcriptional *sipW-lacZ* fusion; 7.8 kb	This study
pUK21	Cloning vector; 2.8 kb; Km^r	(204)
pKW34dT	pUK21 derivative for the disruption of *sipW*; 6.6 kb Km^r ; Tc^r	This study

Bacterial strain	Genotype	Reference
E.coli		
MC1061	F^-; araD139; Δ (ara-leu)7696; Δ (lac)X74; galU; galK; hsdR2; mcrA; mcrB1; rspL	(173)
B. subtilis		
8G5	trpC2; tyr; his; nic; ura; rib; met; ade; lacks the sipP genes	(205)
8G5 sipS (ΔS)	like 8G5; rib^+; sipS	(104)
8G5 sipT-Cm (ΔT)	like 8G5; sipT; Cm^r	(105)
8G5 sipT-Sp	like 8G5; sipT; Sp^r	This study
8G5 sipU	like 8G5; sipU	(105)
8G5 sipV-Em	like 8G5; sipV; Em^r	(105)
8G5 sipV-Sp	like 8G5; sipV; Sp^r	This study
8G5 sipW-Tc (ΔW)	like 8G5; sipW; Tc^r	This study
ΔSU	like 8G5; rib^+; sipS; sipU	This study
ΔSV	like 8G5; rib^+; sipS; sipV; Em^r	This study
ΔSW	like 8G5; rib^+; sipS; sipW; Tc^r	This study
ΔSUV	like 8G5; rib^+; sipS; sipU; sipV; Em^r	This study
ΔSUW	like 8G5; rib^+; sipS; sipU; sipW; Tc^r	This study
ΔSVW	like 8G5; rib^+; sipS; sipV; Em^r; sipW; Tc^r	This study
ΔSUVW	like 8G5; rib^+; sipS; sipU; sipV; Em^r; sipW; Tc^r	This study
ΔTU	like 8G5; sipT; Cm^r; s ipU	This study
ΔTV	like 8G5; sipT; Cm^r; sipV; Em^r	This study
ΔTW	like 8G5; sipT; Cm^r; sipW; Tc^r	This study
ΔTUV	like 8G5; sipT; Cm^r; sipU; sipV; Em^r	This study
ΔTUW	like 8G5; sipT; Cm^r; sipU; sipW; Tc^r	This study
ΔTUVW	like 8G5; sipT; Cm^r; sipU; sipV; Sp^r; sipW; Tc^r	This study
ΔUV	like 8G5; sipU; sipV; Em^r	This study
ΔUW	like 8G5; sipU; sipW; Tc^r	This study
ΔVW	like 8G5; sipV; Sp^r; sipW; Tc^r	This study
ΔST (pGDL41)	like 8G5; rib^+; sipT; Cm^r; contains pGDL41	This study
ΔST (pS-D146A)	like 8G5; rib^+; sipT; Cm^r; contains pS-D146A	This study
ΔSTxS	like 8G5; rib^+; sipT; Sp^r; PxylA-sipS; amyE; Cm^r	This study
ΔSTxS-D146A	like 8G5; rib^+; sipT; Sp^r; PxylA-sipS-D146A; amyE; Cm^r	This study

Table 4.I (*continued*).

Bacterial strain	Genotype	Reference
8G5 :: pGDE22	8G5 carrying pGDE22 (sipS-lacZ) in the chromosome; Cm^r	(104)
8G5 :: pLGT207	8G5 carrying pLGW207 (sipT-lacZ) in the chromosome; Cm^r	(105)
8G5 :: pLGU202	8G5 carrying pLGU202 (sipU-lacZ) in the chromosome; Cm^r	(105)
8G5 :: pLGV201	8G5 carrying pLGV201 (sipV-lacZ) in the chromosome; Cm^r	(105)
8G5 :: pLGW201	8G5 carrying pLGW201 (sipW-lacZ) in the chromosome; Cm^r	This study
8G5 degS-degU	like 8G5; degS-degU; Km^r	(104)
8G5 degU32(Hy)	like 8G5; degU32(Hy); Km^r	(104)
8G5 :: pGDE22 degU32(Hy)	Derivative of 8G5::pGDE22; degU32(Hy); Cm^r; Km^r	(104)
8G5 :: pLGT207 degU32(Hy)	Derivative of 8G5::pLGT207; degU32(Hy); Cm^r; Km^r	This study
8G5 :: pLGU202 degU32 (Hy)	Derivative of 8G5::pLGU202; degU32(Hy); Cm^r; Km^r	This study
8G5 :: pLGV201 degU32(Hy)	Derivative of 8G5::pLGV201; degU32(Hy); Cm^r; Km^r	This study
8G5 :: pLGW201 degU32(Hy)	Derivative of 8G5::pLGW201; degU32(Hy); Cm^r; Km^r	This study

Plasmid pKTH10-BT was constructed in three steps. First, the *amyQ* gene was amplified by PCR with the primers amyQ5' (5'-GGC CTA GGA TCC TAA GAG AGG GAG AGG AAA CAT GAT-3') and amyQ3' (5'-GGC CAT GTC GAC AGT ACT GCA TGC TTA TCT AGA TTT CTG AAC ATA AAT GGA GAC-3') using pKTH10 as a template. The amplified fragment was cleaved with *Bam*HI and *Sal*I, and cloned into the corresponding sites of pBR322, resulting in pBR322*amyQ*. Second, the sequences specifying the biotinylation domain (PSBT) of the *P. shermanii* transcarboxylase 1.3 S subunit were amplified by PCR with the primers PSBT1 (5'-GGC CAT GAG CTC TCT AGA GTC GCC GGT AAG GCC GGA GAG-3') and PSBT2 (5'-GAA TTC GCA TGC GCG TCA GCC GAT CTT GAT GAG ACC-3') using plasmid pCY66 (199) as a template. The amplified fragment was cleaved with *Xba*I and *Sph*I, and cloned into the corresponding sites of pBR322*amyQ*, resulting in pBR322*amyQ*-BT. Third, the *amyQ-PSBT* gene fusion was amplified by PCR with the primers amyQ-BT5' (5'-GGC CAT AAG CTT TAA TCA ATC CGT GTT TGA TGT TCC-3') and amyQ-BT3' (5'-GGC CAT GGA TCC GCG TCA GCC GAT CTT GAT GAG ACC-3'), using pBR322*amyQ*-BT as a template. The amplified fragment was cleaved with *Hin*dIII and *Bam*HI, and ligated into the corresponding sites of pKTH10, resulting in pKTH10-BT.

Plate assay for the processing of the (A13i)-β-lactamase precursor

The plate assay for the processing of the hybrid precursor pre(A13i)-β-lactamase was carried out as described by van Dijl *et al*. (103).

Pulse-chase protein labeling, immunoprecipitation, SDS-PAGE and fluorography

Pulse-chase labeling of *B. subtilis*, immunoprecipitation, SDS-PAGE and fluorography were performed as described previously (164;165). [^{14}C]-Methylated molecular weight markers were from Amersham International. Relative amounts of precursor and mature forms of secreted proteins were estimated by film scanning with an LKB ultroscan XL laser densitometer.

Western blot analysis

Western blotting was performed as described by Kyhse-Andersen (184). After separation by SDS-PAGE, proteins were transferred to Immobilon-PVDF membranes (Millipore Corporation). To detect the α-amylase AmyQ, (A13i)-β-lactamase, or SipS, *B. subtilis* cells were separated from the growth medium by centrifugation (5 min, 12.000 rpm, room temperature), and samples for SDS-PAGE were prepared as described by van Dijl *et al*. (192). AmyQ, (A13i)-β-lactamase, or SipS were visualized with specific antibodies and horseradish peroxidase-anti-rabbit-IgG conjugates (Amersham International). A streptavidin-horseradish peroxidase conjugate (Amersham International) was used to detect biotinylated AmyQ-PSBT.

β-galactosidase activity assay

Overnight cultures were diluted 100-fold in fresh medium and samples were taken at hourly intervals for OD600 readings and β-galactosidase activity determinations. The assay and the calculation of β-galactosidase units (expressed as units per OD600) were carried out as described by Miller (200).

Results

SipW belongs to the sub-family of ER-type SPases

To make an inventory of the known type I SPases, database searches were performed with various P- and ER-type SPases. Unexpectedly, similarity searches with the ER-type SPase of *M. jannaschii* showed that the *yqhE* gene of *B. subtilis* (GenBank accession #D84432) specifies a protein with a high degree of sequence similarity to the known ER-type SPases from archaea and eukaryotes. One of the SPases of *A. fulgidus* (GenBank locus AF1655) exhibited the highest degree of similarity with the deduced YqhE protein sequence (57% identical residues and conservative replacements in 131 residues). This observation strongly suggested that the *yqhE* gene specifies an ER-type SPase of *B. subtilis*. By analogy to other SPase-encoding genes of bacilli, we therefore renamed the *yqhE* gene

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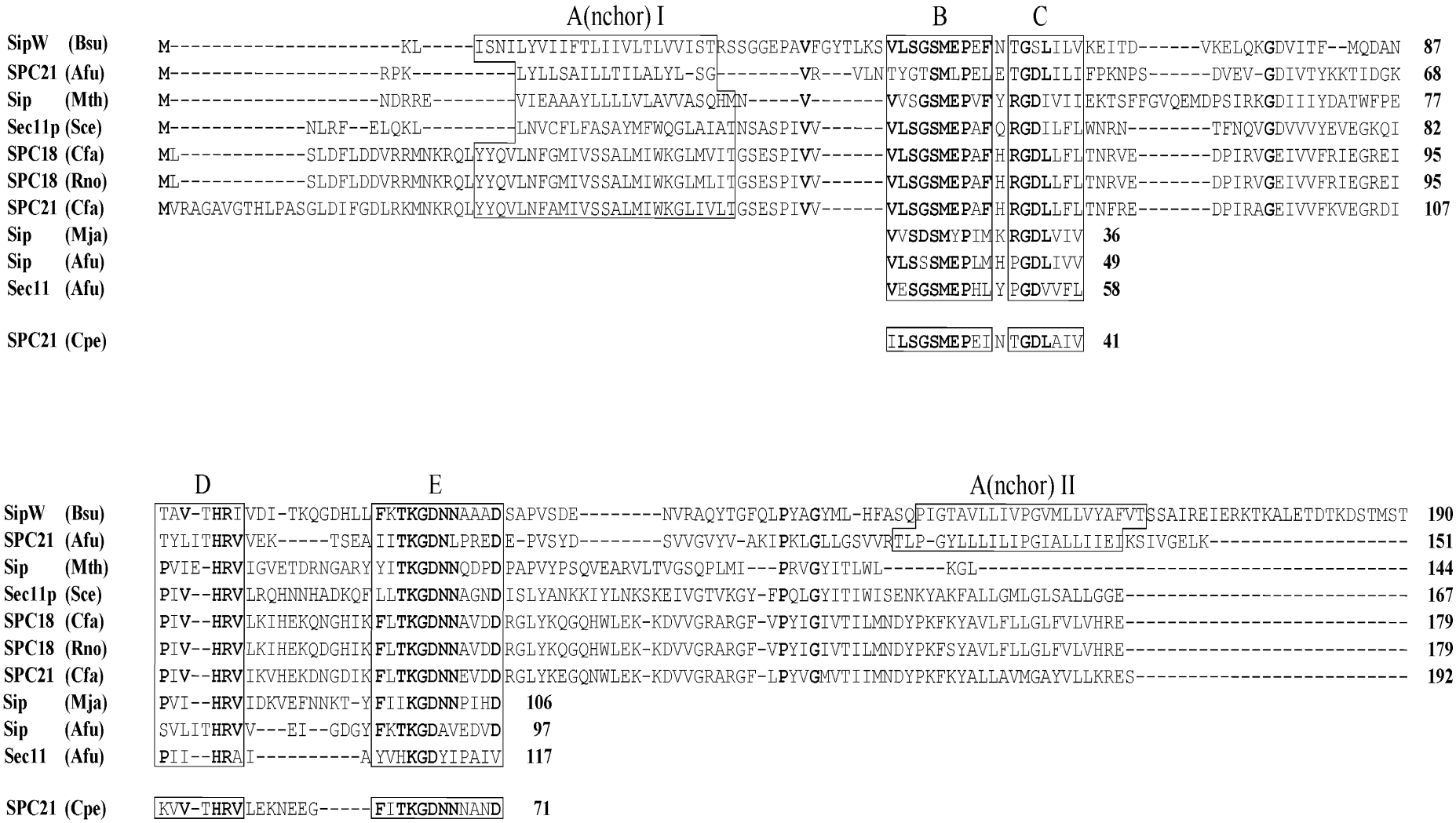
A(nchor) I
B
C
SipW (Bsu) M----------KL-----ISNILYVIIFTLIIVLTLVVISTRSSGGEPAVFGYTLKSVLSGSMEPEFNTGSLILVKEITD------VKELQKGDVITF--MQDAN 87
SPC21 (Afu) M----------RPK----------LYLLSAILLTILALYL-SG--------VR---VLNTYGTSMLPELETGDLILIFPKNPS------DVEV-GDIVTYKKTIDGK 68
Sip (Mth) M----------NDRRE--------VIEAAAYLLLLVLAVVASQHMN-----V-------VVSGSMEPVFYRGDIVIIEKTSFFGVQEMDPSIRKGDIIIYDATWFPE 77
Sec11p (Sce) M----------NLRF--ELQKL---------LNVCFLFASAYMFWQGLAIATNSASPIVV------VLSGSMEPAFQRGDILFLWNRN--------TFNQVGDVVVYEVEGKQI 82
SPC18 (Cfa) ML----------SLDFLDDVRRMNKRQLYYQVLNFGMIVSSALMIWKGLMVITGSESPIVV------VLSGSMEPAFHRGDLLFLTNRVE-------DPIRVGEIVVFRIEGREI 95
SPC18 (Rno) ML----------SLDFLDDVRRMNKRQLYYQVLNFGMIVSSALMIWKGLMLITGSESPIVV------VLSGSMEPAFHRGDLLFLTNRVE-------DPIRVGEIVVFRIEGREI 95
SPC21 (Cfa) MVRAGAVGTHLPASGLDIFGDLRKMNKRQLYYQVLNFAMIVSSALMIWKGLIVLTGSESPIVV------VLSGSMEPAFHRGDLLFLTNFRE-------DPIRAGEIVVFKVEGRDI 107
Sip (Mja) VVSDSMYPIMKRGDLVIV 36
Sip (Afu) VLSSSMEPLMHPGDLIVV 49
Sec11 (Afu) VESGSMEPHLYPGDVVFL 58
SPC21 (Cpe) ILSGSMEPEINTGDLAIV 41
D
E
A(nchor) II
SipW (Bsu) TAV-THRIVDI-TKQGDHLLFKTKGDNNAAADSAPVSDE--------NVRAQYTGFQLPYAGYML-HFASQPIGTAVLLIVPGVMLLVYAFVTSSAIREIERKTKALETDTKDSTMST 190
SPC21 (Afu) TYLITHRVVEK-----TSEAIITKGDNLPREDE-PVSYD--------SVVGVYV-AKIPKLGLLGSVVRTLP-GYLLLILIPGIALLIIEIKSIVGELK---------- 151
Sip (Mth) PVIE-HRVIGVETDRNGARYYITKGDNNQDPDPAPVYPSQVEARVLTVGSQPLMI---PRVGYITLWL------KGL---------- 144
Sec11p (Sce) PIV--HRVLRQHNNHADKQFLLTKGDNNAGNDISLYANKKIYLNKSKEIVGTVKGY-FPQLGYITIWISENKYAKFALLGMLGLSALLGGE---------- 167
SPC18 (Cfa) PIV--HRVLKIHEKQNGHIKFLTKGDNNAVDDRGLYKQGQHWLEK-KDVVGRARGF-VPYIGIVTILMNDYPKFKYAVLFLLGLFVLVHRE---------- 179
SPC18 (Rno) PIV--HRVLKIHEKQDGHIKFLTKGDNNAVDDRGLYKQGQHWLEK-KDVVGRARGF-VPYIGIVTILMNDYPKFSYAVLFLLGLFVLVHRE---------- 179
SPC21 (Cfa) PIV--HRVIKVHEKDNGDIKFLTKGDNNEVDDRGLYKEGQNWLEK-KDVVGRARGF-LPYVGMVTIIMNDYPKFKYALLAVMGAYVLLKRES---------- 192
Sip (Mja) PVI--HRVIDKVEFNNKT-YFIIKGDNNPIHD 106
Sip (Afu) SVLITHRVV---EI--GDGYFKTKGDAVEDVD 97
Sec11 (Afu) PII--HRAI----------AYVHKGDYIPAIV 117
SPC21 (Cpe) KVV-THRVLEKNEEG-----FITKGDNNNAND 71

Ⓑ

	B	C
	★	
P-type	--sgSM-ptl	r-gd-i###
ER-type	vlsgSMeP-f	r-Gd-l###
	D	E
	★	
P-type	-##-KR##	----GDnr---D
ER-type	p#v-HRv#	f-tKGDnn---d

Ⓒ

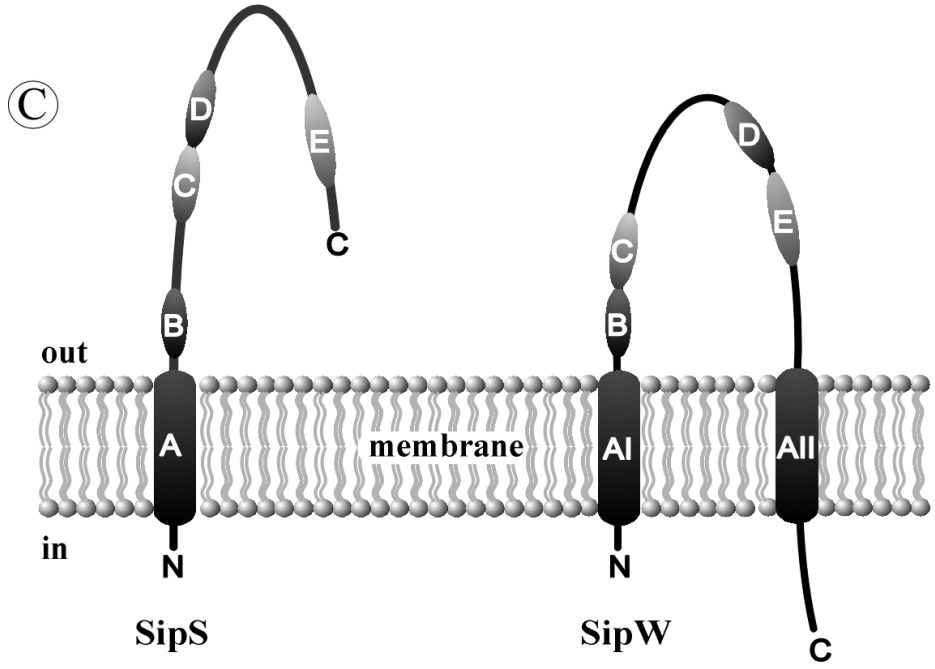

Fig. 4.1. Conserved domains in ER-type SPases from eubacteria, archaea and eukaryotes. ***A*** (page 48), Identification of conserved domains in the ER-type SPases from *B. subtilis* (SipW [Bsu]; GenBank accession #D84432), *A. fulgidus* (SPC21 [Afu], Sec11 [Afu] and Sip [Afu]; GenBank loci AF1657, AF1791 and AF1655, respectively), *M. thermoautotrophicum* (Sip [Mth]; GenBank locus MTH1448), *S. cerevisiae* (Sec11p [Sce]; (193)), *C. familiaris* (SPC18 [Cfa] and SPC21 [Cfa]; (206;207), *Rattus norvegicus* (SPC18 [Rno]; (208), *M. jannaschii* (Sip [Mja], GenBank locus MJU67481), and the partial amino acid sequence of a protein from *Clostridium perfringens* (SPC21 [Cpe]; GenBank accession #X86488). The conserved domains B-E, which are present in all known type I SPases (89;103) are indicated. Because of limited similarities in the regions between conserved domains, only the four conserved domains B-E of Sip (Mja), Sip (Afu), Sec11 (Afu) and SPC21 (Cpe) are shown. Identical amino acids are indicated in bold when present in at least six of the eleven sequences shown. Putative transmembrane segments, indicated with A(nchor) I, or A(nchor) II, were predicted as described by Sipos and von Heijne (20). ***B***, Consensus sequences of the conserved domains B-E of the P- and ER-type SPases. Strictly conserved residues are indicated by upper case letters, conservative substitutions by lower case letters, and conserved hydrophobic residues by a number sign (#). The serine and lysine residues that are critical for the activity of P-type SPases (89) are indicated (*). ***C***, Comparison of the predicted membrane topologies of SipS and SipW of *B. subtilis*. Membrane anchors (A, AI, AII) and the conserved domains B-E are indicated. N, amino-terminus; C, carboxyl-terminus; "in" indicates the cytoplasmic side of the membrane; "out" indicates the extracytoplasmic, cell wall-exposed side of the membrane

sipW (Fig. 4.1a). As previously shown for the ER-type SPases of yeast (*ie*. Sec11p) and *Canis familiaris* (*ie*. SPC18 and SPC21; see: (89)), SipW also shows amino acid sequence similarity with P-type SPases, but this similarity is mainly limited to the conserved domains B-E which are present in all known SPases (Fig. 4.1b). Like the other known ER-type SPases, SipW contains a conserved histidine residue in domain D instead of the conserved lysine residue in domain D of the P-type SPases (Fig. 4.1, a and b). Finally, SipW seems to contain one amino-terminal (AI), and one carboxyl-terminal membrane anchor (AII; Fig. 4.1c), as previously suggested for the P-type SPases of *Rhodobacter capsulatus* and yeast mitochondria (89). The availability of the sequences of SipW of *B. subtilis* and recently identified SPases from archaea allowed the detailed

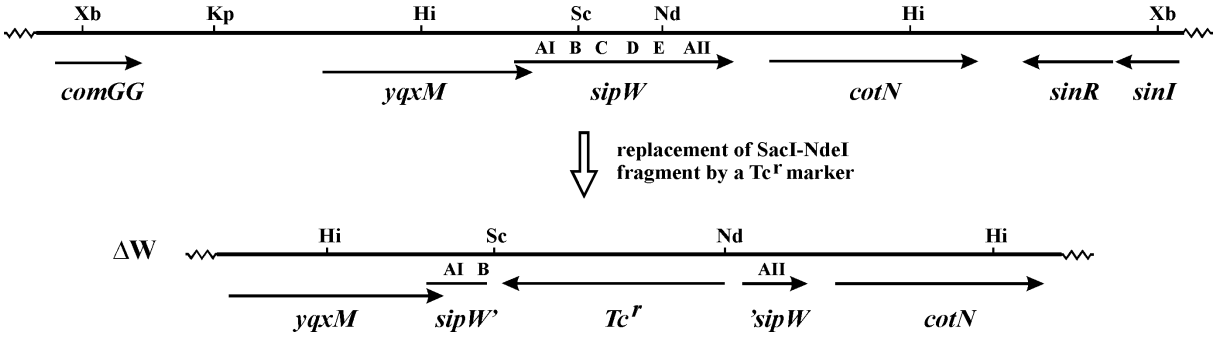

Fig. 4.2. Disruption of the *B. subtilis sipW* gene. Schematic presentation of the construction of *B. subtilis* 8G5 *sipW*-Tc (ΔW). The chromosomal *sipW* gene was disrupted with a Tc^r marker by replacement recombination. To this purpose, *B. subtilis* 8G5 was transformed with the linearized plasmid pKW34dT, in which the 190 bp *Sac*I-*Nde*I fragment (specifying the conserved domains C-E of SipW) was replaced with a Tc^r marker. Only restriction sites relevant for the constructions are shown (Hi, *Hin*dIII; Kp, *Kpn*I; Nd, *Nde*I; Sc, *Sac*I; Xb, *Xba*I). The relative positions of the *comGG*, *yqxM*, *sipW*, *cotN* (later renamed *tasA*; (116)), *sinR* and *sinI* genes are indicated according to (174). Regions of *sipW* specifying the conserved domains B-E and the putative membrane anchors AI and AII are indicated. *sipW* ', 3' truncated *sipW* gene; '*sipW*, 5' truncated *sipW* gene.

comparison of the ER-type SPases, which is documented in Fig. 4.1a. The comparison shows that these enzymes belong to a heterogeneous group of proteins. As previously documented for the P-type SPases, only the domains B-E are conserved in the ER-type SPases (Fig. 4.1, a and b). A striking difference with the P-type SPases is that the domains B and C are separated by only one residue, and the domains D and E are separated by only two to eleven residues (Fig. 4.1, a and c). The corresponding domains of the P-type SPases are separated by 19 to 42, and 23 to 118 residues, respectively (89). Finally, the number of putative membrane spanning domains seems to vary in the ER-type SPases. All known enzymes of this sub-family have one amino-terminal membrane anchor (AI, Fig. 4.1a). In addition, some of these SPases, such as SipW of B. subtilis and SPC21 of A. fulgidus, seem to have a carboxyl-terminal membrane anchor (AII, Fig. 4.1, a and c), and a second SPase of A. fulgidus (Sip [Afu]) seems to have even three carboxyl-terminal membrane anchors (data not shown).

Two quadruple SPase mutants of B. subtilis *are viable*

To determine whether SipW is required for growth or viability of the cell, an internal *Sac*I-*Nde*I fragment of the chromosomal *sipW* gene, specifying the conserved domains C-E, was replaced with a tetracycline resistance (Tc^r) marker (schematically shown in Fig. 4.2). As previously shown for strains lacking intact *sipS*, *sipT*, *sipU,* or *sipV* genes (104;105), the disruption of the *sipW* gene had no detectable effect on cell growth or viability (data not shown).

To investigate which SPases are needed for growth and cell viability, various chromosomal *sip* gene deletions or disruptions were combined. The double, triple and quadruple *sip* mutants obtained are listed in Table 4.I, which shows that most *sip* gene mutations could be combined, with one exception: it was not possible to construct strains lacking both *sipS* and *sipT*. Interestingly, the quadruple mutants jointly lacking *sipS*, *sipU*, *sipV,* and *sipW* (*B. subtilis* ΔSUVW), or *sipT, sipU*, *sipV,* and *sipW* (*B. subtilis* ΔTUVW) showed similar growth rates as the parental strain *B. subtilis* 8G5, irrespective of the growth medium used (data not shown). Thus, it seems that the secretory precursor processing machinery is functionally redundant, and that one SPase (*ie*. SipS or SipT) is sufficient for growth and cell viability of *B. subtilis*.

Functional SipS is essential for viability of a B. subtilis *strain lacking SipT*

To evaluate the importance of SipS and SipT, a strain was constructed, which produces a temperature-sensitive (*ts*) SipS mutant protein, but lacks wild-type SipS and SipT. To this purpose, we first selected *ts* variants from a large collection of previously constructed site-specific mutants of SipS (113). By using a plate assay for SipS activity (103), five *ts* mutants were identified, which are active at 30°C, but not at 48°C (Table 4.II). These contain the L74A, Y81A, R84A, R84H, or D146A mutations, respectively.

In a second step, *B. subtilis* 8G5 *sipS*, which lacks the chromosomal *sipS* gene (104), was transformed with plasmids specifying the L74A, Y81A, R84A, R84H, or D146A SipS mutant proteins. In a third and final step, the *sipT* gene of the latter transformants was disrupted with a chloramphenicol resistance (Cm^r) marker, as previously described (105). The resulting mutant strains (ΔST $sipS^{ts}$) showed no growth defects at 30°C, 37°C and 42°C (data not shown). However, after a temperature shift to 48°C during the exponential growth phase, all five ΔST $sipS^{ts}$ strains stopped growing when they reached the transition phase between exponential and post-exponential growth and, upon continued incubation at 48°C, they even started to lyse. In contrast, the control strain ΔST (pGDL41), producing wild-type SipS, continued to grow in the post-exponential growth phase (Fig. 4.3a). Because all ΔST $sipS^{ts}$ mutant strains showed similar characteristics with respect to growth and cell viability, only one of these strains (*ie.* ΔST [pS-D146A]) was selected for further analyses. As shown by Western blotting, the cellular levels of wild-type SipS were not affected by the temperature shift from 37°C to 48°C. In contrast, the cellular levels of the SipS-D146A, which were already reduced at 37°C, were decreased to levels below detection at 48°C (Fig. 4.3b). In summary, these observations show that, for growth and cell viability of *B. subtilis,* functional SipS is required in the absence of SipT.

Accumulation of secretory pre-proteins in the absence of functional SipS and SipT

As a first approach to monitor the effects of the absence of functional SipS and SipT on the processing of secretory pre-proteins, we analyzed the processing of pre(A13i)-β-lactamase. This hybrid precursor is most efficiently processed by SipS and SipT (103;105). As shown in Fig. 4.3, b and c, the disappearance of the SipS-D146A mutant protein in *B. subtilis* ΔST (pS-D146A) at 48°C was paralleled by the accumulation of pre(A13i)-β-lactamase. In contrast, cells of *B. subtilis* ΔST (pGDL41; wild-type SipS) contained much smaller amounts of this precursor and, in addition, they contained the corresponding mature protein (Fig. 4.3c). These findings show that processing of pre(A13i)-β-lactamase is strongly affected in the absence of functional SipS and SipT.

To investigate the effects of depletion of SipS in cells lacking SipT, two strains with a disrupted *sipT* gene were constructed, in which the transcription of *sipS* or *sipS-D146A* is controlled by the xylose-inducible *xylA* promoter. In these strains, denoted ΔSTxS and ΔSTxS-D146A, respectively, the wild-type *sipS* gene was removed from its original locus and the *xylA-sipS* or *xylA-sipS-D146A* constructs were integrated into the *amyE* locus (Fig. 4.4a).

Unexpectedly, even in the absence of xylose, both strains showed normal growth at 37 °C and 42°C, suggesting that the *xylA* promoter was not completely repressed in the absence of xylose (data not shown). In fact, the inhibition of growth and subsequent cell lysis was only observed for *B. subtilis* ΔSTxS-D146A upon incubation at 48°C in the absence of xylose (data not shown), similar to *B. subtilis* ΔST (pS-D146A) (Fig. 4.3b). As shown by Western blotting experiments with cells in the post-exponential growth phase, the cellular levels of SipS or SipS-D146A in *B. subtilis* ΔSTxS and ΔSTxS-D146A, respectively, depended on the presence or absence of xylose. Compared to the parental strain *B. subtilis* 8G5, *B. subtilis* ΔSTxS contained slightly reduced levels of SipS in the absence of xylose, whereas SipS was about 5-fold overproduced in the presence of xylose. In contrast, in *B. subtilis* ΔSTxS-D146A, only very low levels of SipS-D146A were detectable in the

Table 4.II. Identification of temperature-sensitive SipS mutants

	temperature			
plasmid	**22°C**	**30°C**	**37°C**	**48°C**
pGDL48 (empty vector)	-	-	-	-
pGDL41 (*sipS*)	+	+	+	+
pS-L74A	+	+	+/-	-
pS-Y81A	-	+	-	-
pS-R84A	+	+	-	-
pS-R84H	+	+	-	-
pS-D146A	+	+	-	-

A plate (halo-)assay for the processing of the hybrid precursor pre(A13i)-β-lactamase (103) was used to examine the temperature-sensitivity of mutant SipS proteins. *E. coli* MC1061 strains containing plasmids that specify wild-type SipS (pGDL41), or SipS mutant proteins (pS-x), were analyzed for halo formation at 22°C, 30°C, 37°C and 48°C respectively. Halo formation is indicated with "+".

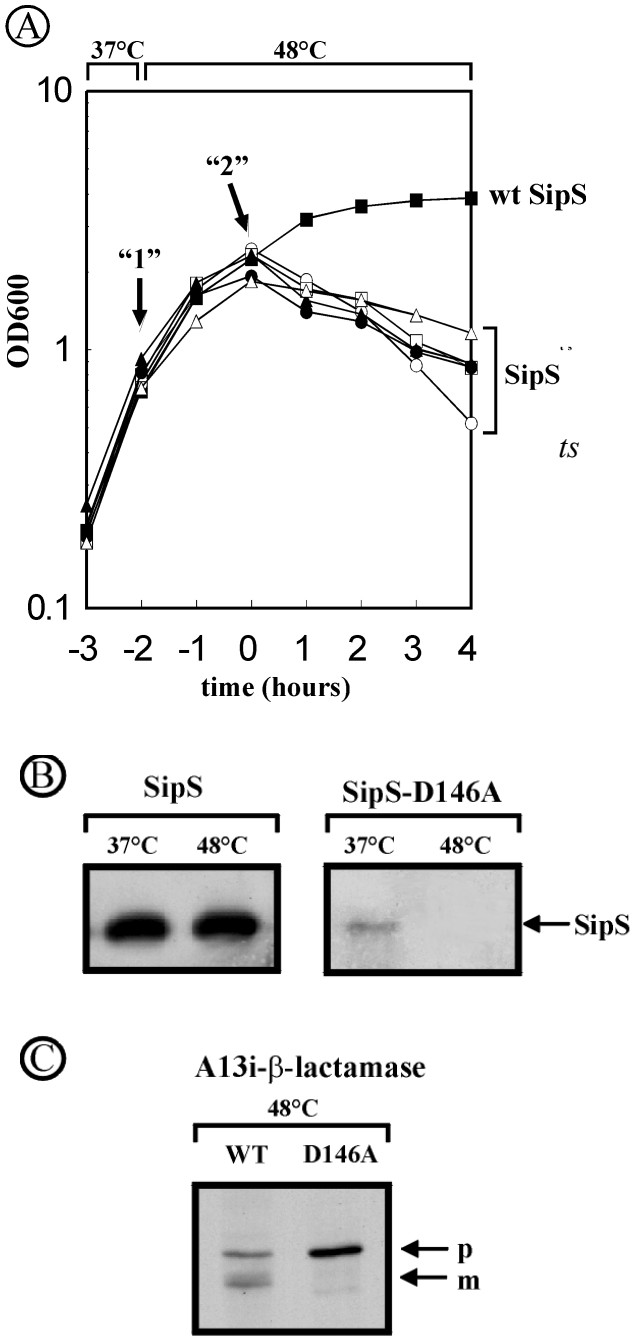

Figure 4.3. Temperature-sensitive growth of *B. subtilis* ΔST containing a *sipS*^{ts} gene. (*A*) Growth curves of *B. subtilis* strains which lack intact chromosomal copies of *sipS* and *sipT*, but contain plasmids specifying the wild-type SipS or temperature-sensitive SipS mutant proteins. The names of the latter plasmids refer to the respective mutation in SipS. ○, *B. subtilis* ΔST (pS-L74A); □, *B. subtilis* ΔST (pS-Y81A); ●, *B. subtilis* ΔST (pS-R84A); ▲, *B. subtilis* ΔST (pS-R84H); Δ, *B. subtilis* ΔST (pS-D146A); ■, *B. subtilis* ΔST (pGDL41; specifies wild-type SipS). Over-night cultures were diluted 100-fold in fresh TY medium and incubated at 37°C. When the cells reached an OD600 of about 0.6, the temperature was shifted to 48°C. Samples were withdrawn shortly before the temperature shift (arrow "1") and two hours after the temperature shift (arrow "2"). Zero time (t=0) indicates the transition point between the exponential and post-expo-nential growth phases. **(*B*)** The presence of wild-type SipS (*B. subtilis* ΔST [pGDL41]) and SipS-D146A (*B. subtilis* ΔST [pS-D146A]) in cells growing at 37°C (arrow "1" in panel *A*) and 48°C (arrow "2" in panel *A*) was analyzed by SDS-PAGE and Western blotting. The position of SipS/SipS-D146A is indicated. **(*C*)** Accumulation of pre(A13i)-β-lactamase in cells of *B. subtilis* ΔST (pGDL41; wild-type SipS) and ΔST (pS-D146A) at 48°C (arrow "2" in panel *A*) was analyzed by SDS-PAGE and Western blotting; p, precursor; m, mature.

presence of xylose, whereas SipS-D146A was not detectable in the absence of xylose (Fig. 4.4b). The latter observation suggests that very small amounts of SipS-D146A are sufficient for growth and cell viability.

To determine the effects of various levels of SipS (D146A) activity on the accumulation of secretory precursor proteins in cells lacking SipT, *B. subtilis* ΔSTxS and ΔSTxS-D146A were transformed with plasmid pKTH10 which specifies the *Bacillus amyloliquefaciens* α-amylase AmyQ. The presence of pKTH10 results in the accumulation of pre-AmyQ, even in wild-type cells (201;209). As shown by Western blotting, cells of *B. subtilis* ΔSTxS-D146A, which were incubated at 48°C in the absence of xylose, accumulated strongly increased amounts of pre-AmyQ (Fig. 4.4c). In contrast, at 37°C, cells of the latter strain accumulated similar amounts of pre-AmyQ as *B. subtilis* ΔSTxS, producing close to wild-type levels of SipS (37°C and 48°C; Fig. 4.4c), or overproducing SipS (data not shown). Taken together, our observations demonstrate that the

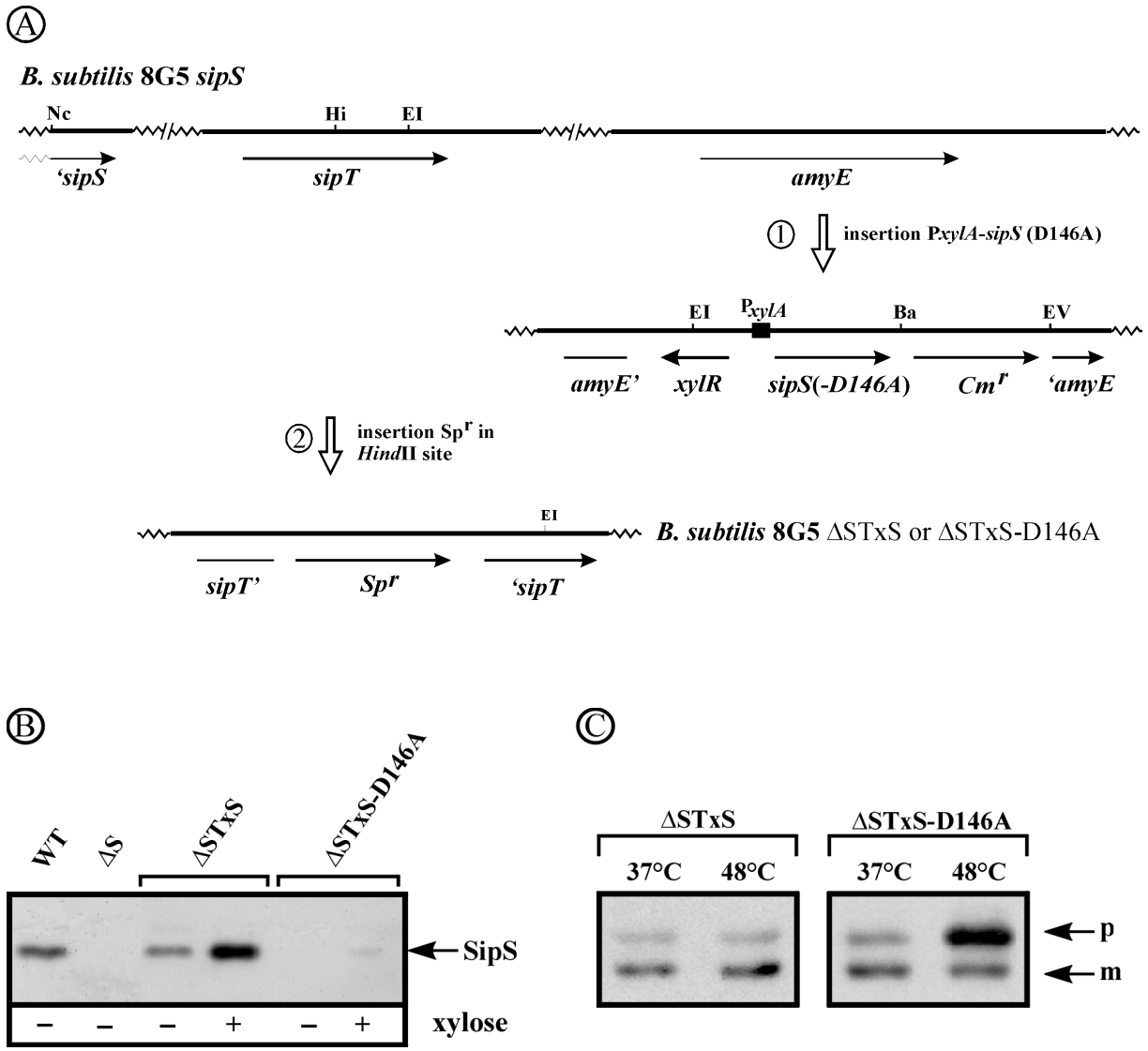

Figure 4.4. Accumulation of pre-AmyQ in *B. subtilis* cells lacking functional SipS and SipT. ***A***, Schematic presentation of the construction of *B. subtilis* ΔSTxS and ΔSTxS-D146A. For this purpose, the *sipS* and *sipS-D146A* genes were amplified by PCR with the primers EX-1 (5'-ACTCTAGATGCCATTGAAATAGACC-3') and EX-2 (5'-ATGGATCCTTGTTTAAAACTTGG-3') using the plasmids pGDL41 and pS-D146A as templates. The amplified fragments were cleaved with *Xba*I and *Bam*HI and ligated to the *Spe*I-*Bam*HI sites of the plasmid pX (202), resulting in pXS and pXS-D146A, respectively. Next, both plasmids were linearized and used to transform *B. subtilis* 8G5 *sipS.* Thus, the *amyE* gene was replaced with the xylose-inducible *sipS* or *sipS-D146A* genes and a Cm^r marker. The inactivation of the *amyE* gene was tested and confirmed by lack of halo formation upon growth on plates containing 1% starch. Finally, the chromosomal *sipT* gene was disrupted with a Sp^r marker by homologous recombination, resulting in the strains ΔSTxS (*sipS, sipT*-Sp, P*xylA-sipS,* Cm^r) and ΔSTxS-D146A (*sipS, sipT*-Sp, P*xylA-sipS-D146A,* Cm^r). Only restriction sites relevant for the constructions are shown (Ba, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; Hi, *Hin*dII; Nc, *Nco*I). '*sipS*, 5' truncated *sipS* gene (104); *amyE*', 3' truncated *amyE* gene; '*amyE*, 5' truncated *amyE* gene; *sipT*', 3' truncated *sipT* gene; '*sipT*, 5' truncated *sipT* gene; *xylR*, gene specifying the XylR repressor protein. ***B***, Detection of wild-type SipS or SipS-D146A in cells of *B. subtilis* 8G5 (WT), 8G5 *sipS* (ΔS), ΔSTxS and ΔSTxS-D146A. Cells were grown in TY medium at 37°C in the presence (+) or absence (-) of 1% xylose, and samples for SDS-PAGE and Western blotting were withdrawn four hours after the transition from exponential to post-exponential growth. ***C***, Accumulation of pre-AmyQ in cells of *B. subtilis* ΔSTxS and ΔSTxS-D146A. Overnight cultures were diluted 100-fold in fresh TY medium without xylose and incubated at 37°C. When the cells reached an OD600 of about 0.6, the temperature was shifted to 48°C. Samples withdrawn shortly before (t=-1; see Fig. 4.3a), and 30 min after the temperature shift (t=-0.5) were analyzed by SDS-PAGE and Western blotting. p, precursor; m, mature.

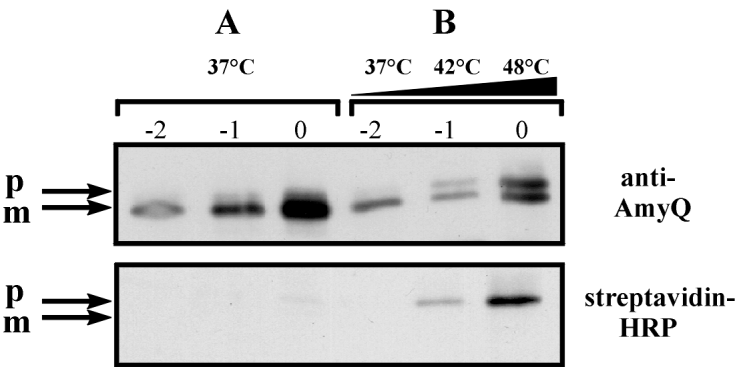

Figure 4.5. Accumulation of biotinylated pre-AmyQ-PSBT in the absence of functional SipS and SipT. An overnight culture of *B. subtilis* ΔSTxS-D146A (pKTH10-BT) was diluted 100-fold in fresh TY medium without xylose and incubated at 37°C. When the culture reached an OD600 of about 0.6, a sample (t=-2) was taken. Subsequently, the culture was divided in two equal moieties A and B. Culture A was kept for two hours at 37°C and samples were taken at t=-1 and t=0. Culture B was incubated for one hour at 42°C and, after a sample (t=-1) had been taken, incubation was continued for one hour at 48°C before a second sample (t=0) was taken. Cells were collected by centrifugation and analyzed by SDS-PAGE and Western blotting. The presence of AmyQ-PSBT was visualized with specific antibodies. Biotinylated pre-AmyQ-PSBT was visualized with streptavidin horseradish peroxidase (HRP). p, precursor; m, mature.

presence of SipS and SipT is required for efficient processing of pre-AmyQ, and that in cells lacking SipT, very small amounts of SipS, which are below the level of detection, are sufficient to prevent the accumulation of pre-AmyQ at high levels.

Impaired translocation of pre-AmyQ in cells lacking functional SipS and SipT

To investigate whether SipS and SipT depletion affects the translocation of pre-AmyQ, the sequences specifying the biotin-accepting domain of a transcarboxylase from *Propionibacterium shermannii* (PSBT; (210)) were fused to the 3' end of the *amyQ* gene on pKTH10. The resulting plasmid pKTH10-BT was used to transform *B. subtilis* ΔSTxS-D146A and, subsequently, the accumulation of biotinylated (pre-)AmyQ-PSBT was determined. The rationale of this experiment is that pre-AmyQ-PSBT can only be biotinylated by the cytoplasmic biotin-ligase if the PSBT domain folds into its native three-dimensional structure in the cytoplasm. This will only happen if the translocation of pre-AmyQ-PSBT across the membrane is impaired. As shown in Fig. 4.5a, processing of pre-AmyQ-PSBT occurred efficiently in cells of *B. subtilis* ΔSTxS-D146A growing at 37°C in the absence of xylose, as only small amounts of pre-AmyQ-PSBT were detectable. Under these conditions, mature non-biotinylated AmyQ-PSBT was secreted into the growth medium (data not shown). Similar results were obtained with the parental strain 8G5, both at 37°C and 48°C (data not shown). In contrast, high levels of pre-AmyQ-PSBT accumulated in cells of *B. subtilis* ΔSTxS-D146A incubated in the absence of xylose at 42°C (permissive temperature for growth) or 48°C (non-permissive temperature) (Fig. 4.5b, upper panel). As demonstrated with a streptavidin horseradish peroxidase conjugate, significant amounts of the accumulating pre-AmyQ-PSBT were biotinylated, showing that the translocation of these precursor molecules was impaired (Fig. 4.5b, lower panel). Compared to cells of *B. subtilis* ΔSTxS-D146A which were grown in the absence of xylose at 37°C, cells grown at 42°C or 48°C accumulated significantly reduced levels of mature AmyQ-PSBT; although mature AmyQ-PSBT was produced in the absence of xylose at 42°C or 48°C, it was not detectably biotinylated (Fig 4.5, a and b).

Relative contributions of SipU, SipV and SipW to the processing of pre-AmyQ

We have previously reported that the processing of pre-AmyQ is stimulated in the absence of SipS, and retarded in the absence of SipT, indicating that the production of SipS interferes with pre-AmyQ processing, and that this precursor is a preferred substrate of SipT (105). To examine the relative contributions of SipU, SipV and SipW to the processing of pre-AmyQ in the absence of SipS or SipT, Western blotting and pulse-chase labeling experiments were performed with strains lacking multiple SPases. The parental strain 8G5 was used as a control. As the results obtained by pulse-chase labeling confirmed those obtained by Western blotting, only the latter are documented. As shown in Fig. 4.6a, cells of *B. subtilis* ΔS, or ΔSU, accumulated only very low levels of pre-AmyQ. Similar results were obtained with cells of *B. subtilis* ΔSV and ΔSW (data not shown). The level of pre-AmyQ was slightly increased in cells of *B.*

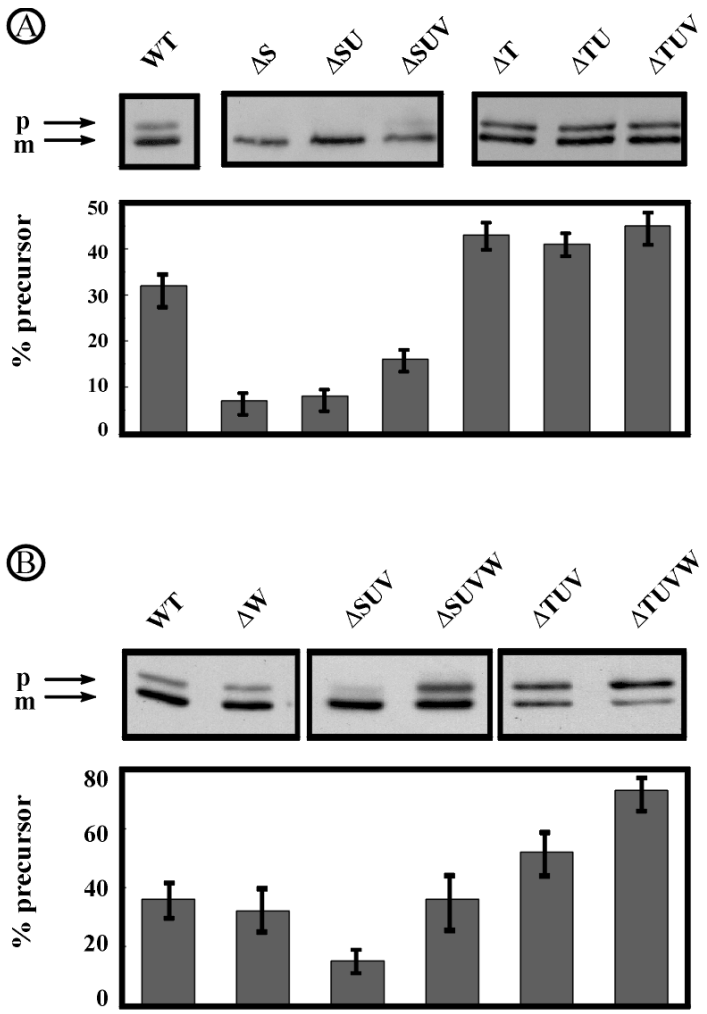

Figure 4.6. Relative contribution of SipU, SipV and SipW to pre-AmyQ processing in the absence of SipS or SipT. ***A***, The accumulation of pre-AmyQ in pKTH10-transformed cells of the parental strain *B. subtilis* 8G5 (WT) and in pKTH10-transformed cells lacking SipS (ΔS), SipS and SipU (ΔSU), SipS, SipU and SipV (ΔSUV), SipT (ΔT), SipT and SipU (ΔTU), or SipT, SipU and SipV (ΔTUV), was analyzed by SDS-PAGE and Western blotting. Samples were taken from overnight cultures in TY medium. p, precursor; m, mature. Relative amounts of precursor and mature forms of AmyQ were determined by densito-meter scanning of films. The average values of three independent experiments are shown, and the standard deviation is indicated by error bars. ***B***, The accumulation of pre-AmyQ in pKTH10-transformed cells of the parental strain *B. subtilis* 8G5 (WT), and pKTH10-transformed cells lacking SipW (ΔW), SipS, SipU and SipV (ΔSUV), SipS, SipU, SipV and SipW (ΔSUVW), SipT, SipU and SipV (ΔTUV), or SipT, SipU, SipV and SipW (ΔTUVW), was analyzed by SDS-PAGE and Western blotting. Samples were prepared from cells grown in TY medium until the early post-exponential growth phase (t=4). Relative amounts of precursor and mature forms of AmyQ are indicated as in *A*.

subtilis ΔSUV, but remained lower than in the parental strain (Fig. 4.6a). The levels of pre-AmyQ accumulation in *B. subtilis* ΔSUW and ΔSVW were similar to those in *B. subtilis* ΔSUV (data not shown). Compared to *B. subtilis* ΔT, which accumulates more pre-AmyQ than the parental strain, cells of *B. subtilis* ΔTU and ΔTUV showed no additional accumulation of pre-AmyQ (Fig. 4.6a), and similar results were obtained with *B. subtilis* ΔTV, ΔTW, and ΔTUW (data not shown). The levels of pre-AmyQ in cells of *B. subtilis* ΔSUV or ΔTUV could only be further increased by disruption of the *sipW* gene, suggesting that SipW is actively involved in the processing of pre-AmyQ (Fig. 4.6b). In conclusion, these observations show that the accumulation of pre-AmyQ in cells lacking either SipS or SipT was significantly exacerbated only by the simultaneous removal of SipU, SipV and SipW.

Transcription of only* sipS *and* sipT *is temporally regulated by the DegS-DegU system

The transcription of the *sipS* and *sipT* genes is temporally controlled, whereas the *sipU* and *sipV* genes are constitutively transcribed (104;105). To analyze the transcription of the *sipW* gene, a transcriptional *sipW-lacZ* fusion was constructed, and introduced into the chromosome of *B. subtilis* 8G5 (schematically shown in Fig. 4.7a). Cells of the resulting strain were grown in TY or minimal medium, and samples withdrawn at hourly intervals were assayed for β-galactosidase activity. The results show that, irrespective of the growth medium used, *sipW* is constitutively transcribed at a low level (only the results for minimal medium are shown in Fig. 4.7b; symbol ▲).

Because it was previously shown that the temporal control of *sipS* transcription is exerted by the DegS-DegU two-component regulatory system (104), which is also required for the transcription of most genes for secreted degradative enzymes

(for review, see (211)), we investigated whether this regulatory system is also involved in the transcription of the *sipT*, *sipU*, *sipV,* and *sipW* genes. To this purpose, the *degU32*(Hy) mutation, which leads to increased transcription of genes for degradative enzymes (211;212) and *sipS* (104), was introduced in strains carrying chromosomal *sipS-lacZ*, *sipT-lacZ*, *sipU-lacZ*, *sipV-lacZ*, or *sipW-lacZ* gene fusions. The resulting strains were grown in minimal medium and samples, withdrawn at hourly intervals, were assayed for β-galactosidase activity. The results show that only the transcription of *sipS* and *sipT* was stimulated in strains containing the *degU32*(Hy) mutation (Fig. 4.7, b and c; indicated with the symbols □ and ■, respectively). By contrast, the *degU32*(Hy) mutation had no effect on the transcription of *sipU*, *sipV*, and *sipW* (Fig. 4.7, b and c; indicated with the symbols ○, ●, and ▲, respectively).

To verify the involvement of the DegS-DegU system in the transcription of the *sipT* gene, Northern hybridization experiments were performed with total RNA from the *B. subtilis* strains 8G5, 8G5 *degU*32(Hy), and 8G5 *degS-degU*. In contrast to *sipS*, for which two transcripts were detectable (104), only one, monocistronic, *sipT*-specific transcript was detected. Compared to the parental strain 8G5, the amount of the *sipT*-specific transcript was about two-fold increased in cells with the *degU*32(Hy) mutation, while it was hardly detectable in cells containing the *degS-degU* deletion (Fig. 4.7d). These findings show that the transcription of the *sipT* gene is controlled by the DegS-DegU system. Consistent with this conclusion, we identified the sequence: 5'-TATGATAAAGTA-3' at a position 46 nucleotides upstream of the translational start of *sipT*. This sequence contains one mismatch and one base insertion compared to a consensus sequence (AMTGAAMAKWW in which K=G or T; M=A or C; W=A or T), which has been implicated in the DegU-dependent control of gene expression (213). Taken together, these results show that the transcription of *sipS* and *sipT* is concerted with that of the genes for secreted degradative enzymes. In contrast, the *sipU*, *sipV*, and *sipW* genes are constitutively transcribed at a low level.

Discussion

Various micro-organisms contain paralogous type I SPases. Interestingly, this seems to be a common feature of Gram-positive eubacteria in particular. For example, *B. amyloliquefaciens* (107;214), *Staphylococcus aureus* (172) and *Streptomyces lividans* (S. Schacht and J. Anné, *personal communication*) contain at least two paralogous SPases of the P-type, whereas *Deinococcus radiodurans* seems to contain at least three of these enzymes (sequences are available *via* ftp://ftp.tigr.org/pub/data/d_radiodurans). Notably, we have identified one ER-type and six P-type SPases in *B. subtilis*. In the present studies, which were aimed at the functional analysis of the chromosomally-encoded SPases of *B. subtilis*, we demonstrate that, in principle, the presence of one SPase (*ie*. SipS or SipT) is sufficient for precursor processing, growth and cell viability. Thus, the secretory precursor processing machinery of *B. subtilis* is functionally redundant, ensuring that this organism can avail of "backup SPases". Under natural conditions, this may be of particular importance for the fitness of *B. subtilis* and other Gram-positive eubacteria, which can secrete large amounts of proteins into the medium as an adaptive response to changes in the environment.

Our results show that SipS and SipT play a major role in the processing of secretory pre-proteins and that, under the conditions tested, the three other chromosomally-encoded SPases (SipU, SipV, and SipW) contribute only to a minor extent to this process. Interestingly, the transcription of *sipS* (104) and *sipT* (this study) is temporally controlled *via* the DegS-DegU two-component regulatory system, in concert with the transcription of the genes for most secretory proteins. Thus, *B. subtilis* can increase its pre-protein processing capacity under conditions of high-level protein secretion in the post-exponential growth phase. Our observation that cells lacking SipS and SipT stop growing, and die in the transition phase between exponential and post-exponential growth, indicates that the potential to increase the processing capacity is important for cell growth and viability. Nevertheless, the production of very small amounts of SipS-D146A, a temperature-sensitive variant of

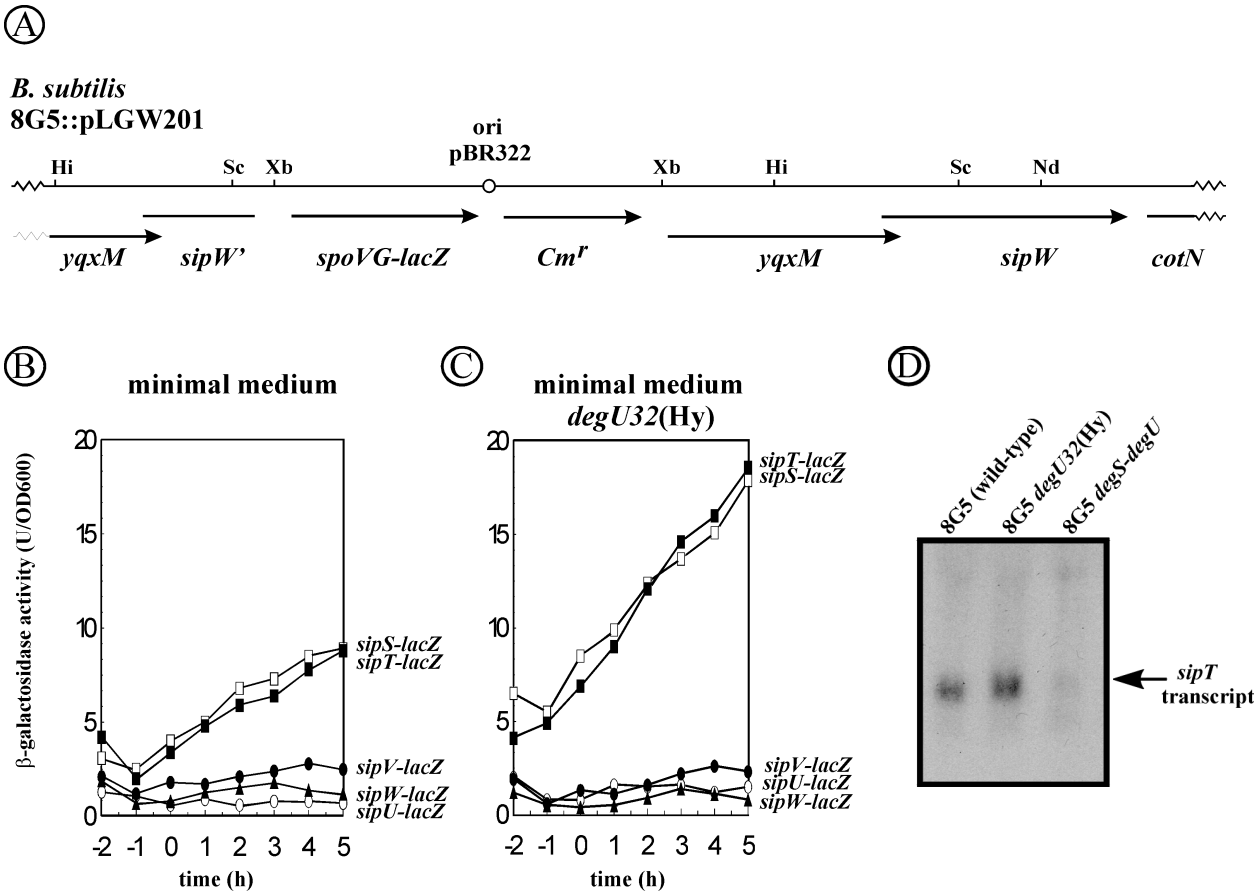

Fig. 4.7. Analysis of the transcription of the *sip* genes of *B. subtilis*. ***A***, Schematic presentation of the *sipW* region on the chromosome of *B. subtilis* 8G5::pLGW201. A transcriptional *sipW-lacZ* gene fusion was constructed with plasmid pLGW200 (203), a chromosomal integration plasmid for *B. subtilis* containing a promoterless *spoVG-lacZ* gene fusion. For this purpose, a fragment comprising the *yqxM* gene and the 5' end of *sipW* was amplified by PCR with the primers lbw-3 (5'-CAT CTA GAT ACA GGA GGT AAG ATA TG-3') and lbw-5 (5'-AGT TCT AGA GTA TTT GCA TCC TGC-3'). Next, the amplified fragment was cloned into the *Xba*I-site of the multiple cloning site upstream of the *spoVG-lacZ* gene fusion on pLGW200, resulting in pLGW201. Finally, the *sipW-lacZ* gene fusion was introduced in the chromosome of *B. subtilis* 8G5 by a Campbell-type integration of pLGW201, resulting in *B. subtilis* 8G5::pLGW201. In the latter strain, the transcription of *lacZ* is directed by the promoter(s) of *sipW*. Only restriction sites relevant for the constructions are shown (Hi, *Hin*dIII; Nd, *Nde*I; Sc, *Sac*I; Xb, *Xba*I), ori pBR322, replication functions of pBR322. ***B***, Time courses of the expression of *sip-lacZ* gene fusions were determined in cells growing in minimal medium at 37°C. β-Galactosidase activities (in Units per OD600) were determined for *B. subtilis* 8G5::pGDE22 (□, *sipS-lacZ*), *B. subtilis* 8G5::pLGT207 (■, *sipT-lacZ*), *B. subtilis* 8G5::pLGU202 (○, *sipU-lacZ*), *B. subtilis* 8G5::pLGV201 (●, *sipV-lacZ*) and *B. subtilis* 8G5::pLGW201(▲, *sipW-lacZ*). Zero time (t=0) indicates the transition point between the exponential and post-exponential growth phases. ***C***, Effects of the *degU32*(Hy) mutation on the expression of *sip-lacZ* gene fusions were determined as in (*B*). To this purpose, strains containing the *sip-lacZ* gene fusions indicated in (*B*) were transformed with chromosomal DNA of *B. subtilis* 8G5 *degU32*(Hy). ***D***, Detection of *sipT*-specific transcripts by Northern hybridization. Equal amounts of total RNA from cells of *B. subtilis* 8G5, 8G5 *degU32*(Hy) and 8G5 *degU-degS* in the transition state were loaded in each lane. A [^{32}P]-dCTP-labeled DNA fragment, amplified by PCR with the primers lbt-9 (5'-AT GAA TTC AGC CCG GTT ATC TCC-3') and lbt-10 (5'-GGA AGT CGA CAT ACG TAC CTG GAA TGG-3'), was used as a probe.

SipS, is sufficient for growth and cell viability. This suggests that the increased processing capacity provided by SipS and SipT relates to, as yet undefined, differences between the specificities of these two SPases and those of SipU, SipV, and/or SipW. This view is supported by the observation that SipU, SipV, and SipW can not complement for the absence of SipS and SipT, even if the corresponding genes are placed on multi-copy plasmids (our unpublished results). In this respect it is important to note that we have previously shown that SipS, SipT, SipU, and SipV have overlapping substrate specificities (105). At present, we do not know the precise function of each of the SPases of *B. subtilis*, but it seems likely that SipS and SipT are required, in particular, for the processing of pre-proteins that are synthesized in the post-exponential growth phase. In contrast, as the *sipU*, *sipV*, and *sipW* genes are transcribed constitutively, the corresponding SPases are most

likely involved in the processing of pre-proteins which are synthesized during all growth phases.

The previously reported presence of SPase-encoding genes on endogenous plasmids (107) seems to give certain strains of *B. subtilis* an additional possibility to increase their capacity for pre-protein processing. The latter hypothesis is supported by our observation that the SPase SipP, specified by plasmid pTA1015, can complement for the absence of SipS and SipT (see Chapter 4). Thus, it seems that SipS, SipT, and SipP have similar substrate specificities.

Dalbey and Wickner (86) have shown that *E. coli* cells depleted of their type I SPase (*ie*. leader peptidase) accumulate pre-proteins on the outer surface of the cytoplasmic membrane. Thus, processing by SPase was shown to be a prerequisite for the release of translocated proteins from the membrane. However, the question whether the accumulation of secretory pre-proteins in cells depleted of SPase would affect protein translocation across the membrane remained unresolved. Our observation that biotinylated pre-AmyQ-PSBT accumulated in *B. subtilis* cells lacking functional SipS and SipT shows that this is indeed the case. Thus, the decreased viability of these cells may be due to impaired protein translocation across the membrane. However, the alternative explanation that certain proteins, which are essential for cell growth and viability, do not reach their correct cellular localization (*eg*. the cell wall) can not be excluded. Interestingly, no biotinylated mature AmyQ-PSBT was produced by cells lacking functional SipS and SipT, suggesting that biotinylated pre-AmyQ-PSBT is not translocated across the membrane of *B. subtilis*. As biotinylated proteins can be transported by the pre-protein translocase of *E. coli* (210), it seems that the pre-protein translocase of *B. subtilis* is more restrictive with respect to (partially) folded, biotinylated pre-proteins than that of *E. coli*.

An intriguing result of the present studies is that *B. subtilis* contains an ER-type SPase (SipW). Thus, *B. subtilis* is the first known eubacterium containing SPases of both the P- and the ER-type. This may not be a peculiarity of *B. subtilis*, as we identified the partial sequence of a SipW/SPC21-like protein from *Clostridium perfringens* by similarity searches in GenBank (accession #X86488). Furthermore, the identification of SipW of *B. subtilis* has at least three interesting implications. Firstly, the presence of ER-type SPases in all three domains of life, suggests that the P-type SPases, which are only present in eubacteria and the organelles of eukaryotes, have evolved from the ER-type SPases. However, the alternative possibilities of convergent evolution of P- and ER-type SPases and/or horizontal transfer of ER-type SPases from archaea or eukaryotes to eubacteria can not be excluded. Secondly, recent studies indicate that the P-type SPases make use of a serine-lysine catalytic dyad (89). In contrast, the catalytic mechanism of the ER-type SPases is presently unclear, because the conserved lysine residue in domain D, which is invoked in the catalytic mechanism of the P-type SPases, is not conserved in the ER-type SPases; instead the ER-type SPases contain a conserved histidine residue at this position (Fig. 4.1b). This could mean that the ER-type SPases use the latter histidine residue for catalysis, for example in a serine-histidine-aspartic acid catalytic triad, like in the classical serine proteases. In addition to the conserved histidine residue in domain D, such a catalytic triad could involve the strictly conserved serine residue in domain B, which is essential for the activity of the P-type SPases, and the strictly conserved aspartic acid residue in domain E. Alternatively, the ER-type SPases could make use of a serine-lysine catalytic dyad, involving the strictly conserved serine and lysine residues in domains B and E, respectively. Thirdly, the ER-type SPases of yeast and higher eukaryotes are part of larger complexes, containing three additional subunits of which the SPC3 or SPC22/23 subunits, respectively, are essential for SPase activity (215-217). None of these additional subunits is conserved in eubacteria or archaea (our unpublished results), suggesting that the ER-type SPases of the latter groups of organisms require no, or very different additional subunits for activity. The identification of the catalytic mechanism of the ER-type SPases, and the identification of their putative partner proteins in eubacteria and archaea are major challenges for future research.

Finally, the present studies on the *sip* gene family of *B. subtilis* have important implications for projects on the functional analysis of the genomes of *B. subtilis* and other organisms, such as *E. coli* and yeast, because the presence of paralogous gene families is a common feature of these microbial genomes. As examplified by the lack of effect of the single disruption of the *sipW* gene, strategies based on single gene disruptions will probably be inadequate to assign functions to many multiplied genes with thus far unidentified functions.

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